

Antithrombin reduces shedding of the endothelial glycocalyx following ischaemia/reperfusion

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Aims Antithrombin is an important inhibitor of the coagulation system, additionally exerting specific anti-inflammatory effects on endothelial cells. Healthy vascular endothelium is coated by the endothelial glycocalyx, diminution of which increases capillary permeability, e.g. after ischaemia. Antithrombin is known to infiltrate the glycocalyx, binding to glycosaminoglycans, and to preserve the glycocalyx after application tumour necrosis factor- α . We investigated the influence of antithrombin on glycocalyx subjected to ischaemia/reperfusion.

Methods and results Isolated guinea pig hearts were perfused with Krebs–Henseleit buffer (KHB). Antithrombin was applied to achieve physiological levels (1 U/mL) before inducing 20 min of ischaemia (37°C). Hearts were reperfused for 20 min at constant flow (baseline perfusion pressure 70 cmH₂O) with KHB or KHB plus 2 g% hydroxyethyl starch (130 kDa). Coronary net fluid filtration was assessed directly by measuring transudate formation on the epicardial surface. Post-ischaemic coronary release of syndecan-1 and heparan sulfate was quantified by ELISA. Hearts were perfusion-fixed to visualize the glycocalyx by electron microscopy. Ischaemia/reperfusion caused degradation of the glycocalyx, enhanced coronary perfusion pressure, and increased vascular permeability. Antithrombin significantly reduced post-ischaemic glycocalyx shedding, coronary perfusion pressure, coronary leak, and tissue oedema formation compared to untreated hearts. Additional application of colloid augmented these actions of antithrombin. Electron microscopy revealed a mostly intact glycocalyx after antithrombin treatment.

Conclusion Antithrombin preserves the endothelial glycocalyx, sustaining the vascular barrier function and reducing interstitial oedema. The potentiated effect of colloid in these hearts suggests that the prevention of shedding should be of functional benefit also *in vivo*.

1. Introduction

Antithrombin III is a physiological inhibitor of serine proteases such as thrombin and elastase and not only inhibits coagulation abnormalities, but reduces inflammatory responses.¹ One such mechanism of action involves promoting endothelial production of prostacyclin by interacting with heparin-like glycosaminoglycans, a vital part of the endothelial glycocalyx.^{2–4} Interaction with the endothelial glycosaminoglycans is also crucial for the potentiation of thrombin inhibition by antithrombin.^{3–5}

It is now realized that a glycocalyx coats every healthy vascular endothelial cell. It consists of a variety of transmembrane and membrane-bound molecules, predominantly syndecan-1 and heparan sulfate.^{6,7} Together with bound plasma proteins and solubilized glycosaminoglycans, the

glycocalyx forms the endothelial surface layer. This latter structure has a functional thickness of $>1 \mu\text{m}$ and is, therefore, mostly as thick as the endothelial cells themselves.^{6,8} Early manifestation of endothelial injury after ischaemia/reperfusion (I/R),⁹ tumour necrosis factor- α (TNF- α)-induced inflammation,¹⁰ or application of heparinase¹¹ has been shown to consist of a disruption of the glycocalyx. Diminution of the endothelial glycocalyx increases capillary permeability, leading to tissue oedema, suggesting the glycocalyx to act as a competent barrier against passage of water and colloids.¹² In the special case of cardiac I/R, functional disturbances at least partly due to disruption of the glycocalyx are impairment of endothelium-dependent coronary vasodilatation, coronary low-reflow, and oedema. Looking at the targets and therapeutic mechanisms of antithrombin, i.e. inhibiting proteases, decreasing inflammatory responses, and interacting with the glycocalyx, indicates a possible further mode of action of this plasma protein: stabilization of the endothelial glycocalyx. We

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have already investigated this possibility in an isolated perfused heart model using TNF- α to degrade the glycocalyx and to alter coronary vascular function related to the state of the glycocalyx. Those experiments revealed a reduced shedding of the glycocalyx if the hearts were pre-treated with antithrombin.¹³

Our present study aimed to show if stabilization of the glycocalyx by antithrombin maintains the physiological endothelial permeability barrier in the face of ischaemic stress and reperfusion damage, thereby mitigating interstitial oedema and post-ischaemic low-reflow.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The study is approved by the officially installed independent Ethics Committee of the State of Bavaria. Licensure of the investigator has been granted by the Government of Upper Bavaria (file No. 209.1/211-2531.3-3/99).

2.1 Heart preparation

Guinea pig hearts were isolated and perfused in a modified Langendorff mode.^{9,12} In brief, male animals (weight 250–300 g) were stunned by cervical dislocation, using a specially designed instrument. Immediately after medial thoracotomy, the hearts were arrested with cold isotonic saline fluid. The aorta was then cannulated and the coronaries perfused *in situ* at constant aortic pressure (70 cmH₂O) with a modified Krebs–Henseleit buffer (KHB) (in mM: 116 NaCl, 23 NaHCO₃, 3.6 KCl, 1.16 KH₂PO₄, 1.2 CaCl₂, 0.58 MgSO₄, 5.4 glucose, 0.3 pyruvate, and 2.8 U/L insulin) gassed with 94.6% O₂ and 5.4% CO₂ at 37°C, pH 7.40 \pm 0.05. Hearts were removed from the thorax and prepared as described previously.¹² The perfusion rate was regulated via peristaltic pump to achieve a constant pressure of 70 cmH₂O during the equilibration phase. The

individual coronary perfusion rate established at the end of the equilibration phase was then maintained for the respective heart throughout the reperfusion. To monitor changes in coronary resistance, perfusion pressure was measured in the aortic feed line by means of a pressure transducer and registered online. Coronary venous effluent draining from the coronary sinus into the right atrium and ventricle was collected from the cannulated pulmonary artery. The level of egress was placed below the apex of the heart to ensure that no pressure-volume work was performed by the right ventricle in transporting coronary effluent. Transudate, a mixture of interstitial and lymphatic fluids appearing on the epicardial surface and directly corresponding to net coronary filtration, was collected over timed intervals as it dripped from the apex of the heart.

2.2 Experimental protocols

Immediately after explantation and preparation of the hearts, an equilibration interval of 15 min was allowed to establish steady-state conditions. Baseline measurements of coronary effluent and transudate were performed in the last 2 min before induction of 20 min of warm (37°C), global stopped-flow ischaemia. Samples of effluent were collected again throughout min 0–5, 5–10, 10–15, and 15–20 after the onset of reperfusion. Transudate samples were collected over 2 min intervals at 0–2, 2–4, 4–6, 6–8, 12–14, and 18–20 min after the start of reperfusion. Therefore, during the first–decisive–minutes of perfusion, all fluids coming from the heart were collected and quantified gravimetrically. Since the rate of transudate formation is pressure-dependent,¹⁴ we related it to the respective perfusion pressure established at the given flow rates in each heart.

The experimental groups are characterized in Figure 1. Global warm ischaemia was induced for 20 min without [Group A ($n = 9$)] or with prior application of antithrombin [Group B ($n = 8$)]. Antithrombin (Kybernin, ZLB Behring, Marburg, Germany) was applied during the entire equilibration phase at a rate of 1 U/mL perfusate (1 U/mL is equivalent to \sim 100% activity in human blood¹³). In Groups C and D (without and with antithrombin, respectively,

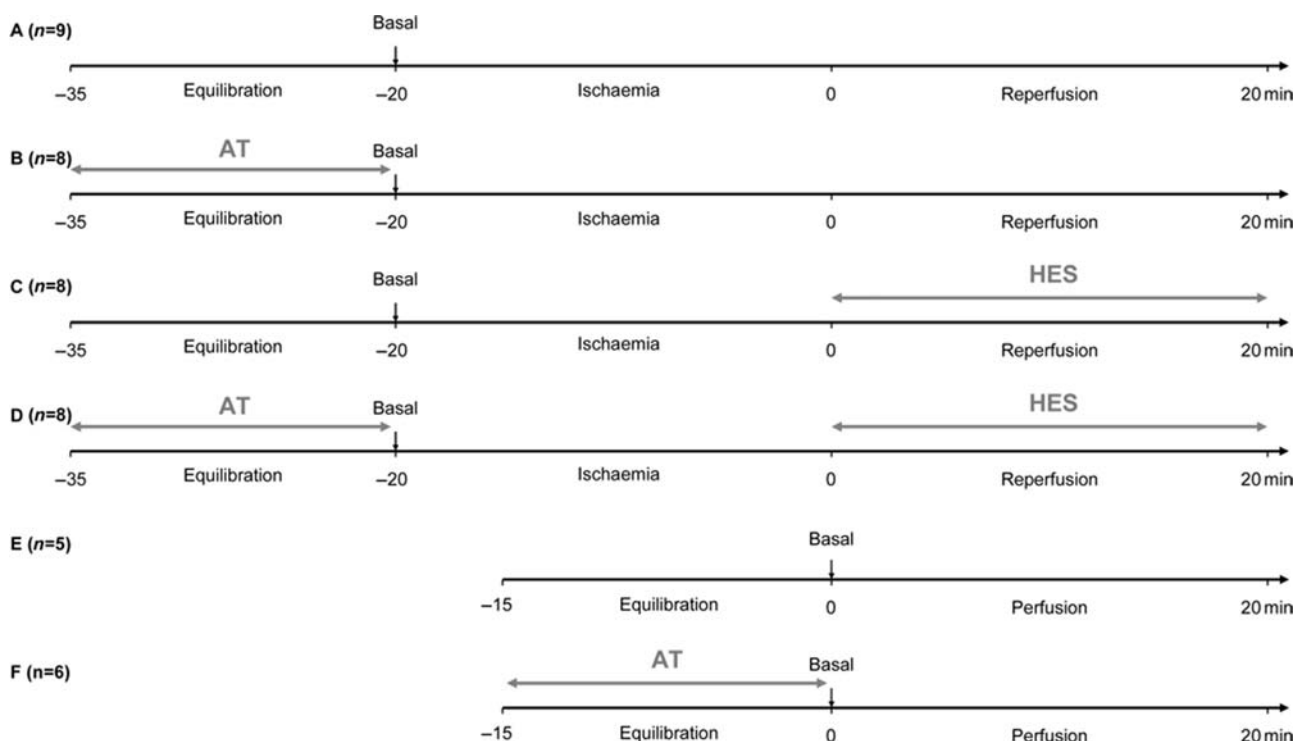


Figure 1 Experimental protocols. Ischaemia is defined as 20 min no-flow warm ischaemia, (re)perfusion as 20 min constant flow perfusion starting with a flow rate that had generated a perfusion pressure of 70 cmH₂O under basal conditions (equilibration). AT, antithrombin III; HES, hydroxyethyl starch.

$n = 8$ each), reperfusion was conducted in the presence of hydroxyethyl starch (HES). This was achieved by infusing 6% HES (molecular weight, 130 000; degree of substitution, 0.4; Fresenius AG, Bad Homburg, Germany) for 20 min into the KHB perfusate at a rate of one-third of the actual, total coronary flow. Over and above that, time-control experiments were performed to evaluate the impact of perfusion without I/R injury, both without [Group E ($n = 6$)] and with application of antithrombin [Group F ($n = 6$)].

At the end of each experiment, both atria and the large vessels were cut away and the ventricles weighed at once (wet weight) and again after 24 hours drying at 60°C (dry weight). This established a wet-to-dry-weight ratio, serving as a quantitative measure for formation of oedema.

2.3 Determination of glycocalyx-components, hydroxyethyl starch, and release of lactate, uric acid, and purines

In all groups, samples of effluent were used for assessing shedding of heparan sulfate and syndecan-1 (CD-138) as described in detail elsewhere.^{9,12} Samples (4 mL) were concentrated to 50 μ L with 10 kDa cut-off ultrafilters (Millipore, Billerica, MA, USA). Aliquots (20 μ L) were used for determining heparan sulfate and syndecan-1. One enzyme-linked immunosorbent assay (ELISA) (Seikagaku Corporation, Tokyo, Japan) was based on two antibodies specific for heparan sulfate-related epitopes, and the other ELISA (Diacclone Research, Besancon, France) employed a solid-phase monoclonal B-B4 antibody and a biotinylated monoclonal B-D 30 antibody raised against syndecan-1. Detection relied on streptavidin-horse-radish peroxidase and tetramethylbenzidine as chromogen in both assays.

Concentrations of HES were quantified in both, the samples of coronary effluent and transudate, using a method described by Forster *et al.*,¹⁹ as modified by Rehm *et al.*¹² Basically, this involves hydrolysis of HES to glucose and subsequent enzymatic/photometric determination of the sugar.

Lactate, purines, and uric acid were determined by high-performance liquid chromatography in samples of coronary effluent. Lactate is an indicator of the severity of ischaemic challenge. Purine release is directly related to the rate of energy consumption and inversely related to the rate of energy production. Uric acid, the end product of enzymatic purine metabolism in the heart, is subject to further oxidative chemical degradation. Thus, hearts under oxidative stress typically release less urate relative to precursor purines than when oxidative stress is mitigated. For lactate, 10 μ L of effluent were applied to a nucleosil 100-5NH₂ 4 \times 250 mm column (Macherey-Nagel, Dueren, Germany); 10 mM NH₄H₂PO₄, pH 3.5, served as eluent. Lactate was detected by its ultraviolet absorbance at 210 nm. Uric acid levels were determined in 10 μ L of coronary effluent sample applied to a 5 μ m C-18 nucleosil 4 \times 250 mm column (Macherey-Nagel). HClO₄/methanol/water at a ratio of 90:6:4 (vol%) served as eluent. Uric acid was detected by its ultraviolet absorbance at 280 nm. In the case of purines, all were transformed first to uric acid and then determined as such. This involved sequential enzymatic conversion of adenosine, inosine, hypoxanthine, and xanthine to urate.¹⁶

2.4 Electron microscopy and immunohistochemistry

Electron microscopy was performed on hearts perfusion-fixed with a lanthanum nitrate glutaraldehyde solution in modification of a method described by Vogel *et al.*¹⁷ For immunohistochemistry, hearts were perfusion fixed and stored in 4% formaldehyde solution for 24 h. Paraffin sections (5 μ m) were stained with monoclonal antibody against human antithrombin III (Sigma Aldrich, St Louis, MO, USA). Controls, in which the primary antibody was replaced with buffer, were treated identically. Histogreen (Linaris, Wertheim, Germany) was used as chromogen.

2.5 Statistical analysis

All data are presented as mean \pm SEM, with n indicating the number of experiments. Comparisons involving two groups were made using the Mann-Whitney U test for independent data or the Wilcoxon test for dependent data. For comparisons of more than two groups, ANOVA on ranks analysis was performed. *Post hoc* tests were performed using the Bonferroni method. A value of $P < 0.05$ was considered to be significant. The statistical software used to conduct the analyses was SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1 Measurement of constitutional parts of the glycocalyx

Small amounts of CD-138 (syndecan-1)-positive material were detected in the effluent of all perfused hearts (basal value 98 ± 22 ng/min/g). As shown in *Figure 2A*, I/R enhanced shedding of syndecan-1 \sim 10-fold. Application of antithrombin significantly decreased shedding at all times (Group A vs. Group B, $P < 0.01$). In fact, values showed no difference to basal shedding rates.

Heparan sulfate was also detected in the effluent of all hearts (basal value 2.4 ± 0.7 μ g/min/g). Ischaemia/reperfusion induced strong, 20-fold heightened shedding of heparan sulfate from the glycocalyx. Although shedding was increased in the first minutes of reperfusion compared with basal values also in Group B ($P < 0.05$; *Figure 2B*), application of antithrombin significantly lowered the release of heparan sulfate throughout reperfusion (Group A vs. Group B, min 0-5: $P < 0.05$; min 5-10, 10-15, and 15-20: $P < 0.01$).

3.2 Electron microscopy and immunohistochemistry

Electron microscopic photographs illustrating the state of the endothelial glycocalyx of coronary vessels are depicted in *Figure 3*, and the measured widths of the structure are provided in *Figure 4*. Time-control hearts possessed a thick glycocalyx with an average width of \sim 400 nm. Only a rudimentary glycocalyx could be visualized in the Groups A and C ($P < 0.01$ vs. time control). On the other hand, a mostly intact glycocalyx, albeit with reduced thickness of about 220 nm ($P < 0.05$ vs. time control), was seen in the antithrombin-treated groups.

Extra hearts were perfused with antithrombin after I/R and then stained with an antibody against human antithrombin III. Immunohistochemistry revealed that for a healthy endothelium with an intact glycocalyx, infused antithrombin presents itself as a ring on the endothelial cells (Group F, *Figure 5*). This rather conspicuous zone indicates that antithrombin is located both on and within the glycocalyx. In vessels of hearts subjected to I/R prior to antithrombin infusion (no study group), antithrombin staining is found in the interstitial tissue and not in the vessel. Obviously, antithrombin extravasates when destruction of the endothelial glycocalyx leads to break-down of the vascular barrier and loss of binding sites at the luminal surface (*Figure 5*).

3.3 Perfusion pressure

Coronary flow rate was adjusted to achieve a constant perfusion pressure of 70 cmH₂O and then maintained

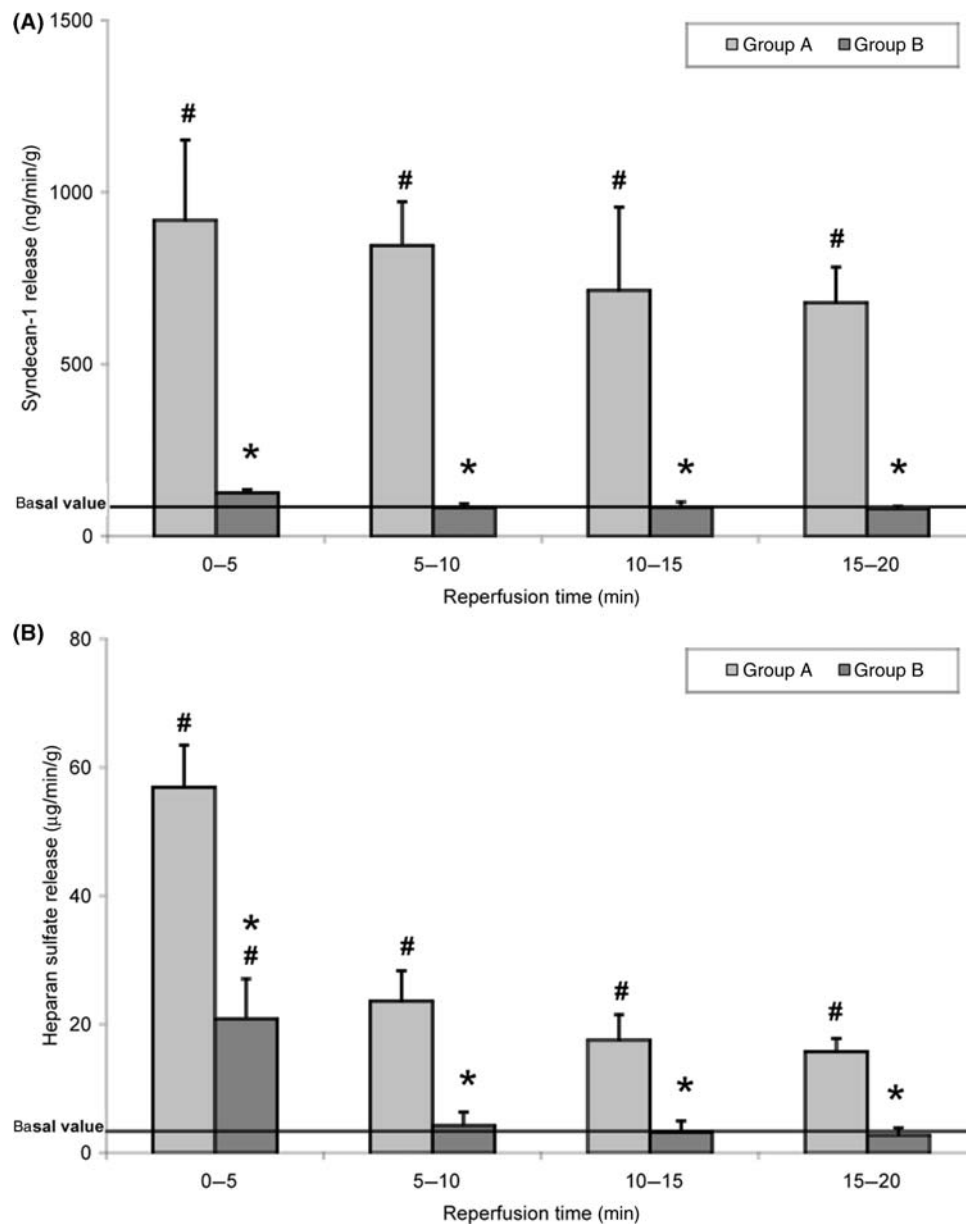


Figure 2 Measurements of constitutional parts of the glycocalyx in coronary venous effluent of heart reperfused without (Group A) or with antithrombin III (Group B). (A) Syndecan-1 release in relation to sampling period during reperfusion *Group A vs. Group B ($P < 0.05$). # $P < 0.01$ vs. basal. (B) Heparan sulfate release in relation to sampling period during reperfusion. *Group A vs. Group B ($P < 0.05$). # $P < 0.01$ vs. basal.

throughout the consecutive reperfusion. Groups showed no flow differences at baseline (A, 8.2 ± 0.5 ; B, 8.5 ± 0.4 ; C, 8.3 ± 0.6 ; D, 8.1 ± 0.5 ; E, 8.3 ± 0.5 ; and F, 8.0 ± 0.3 mL/min; all comparisons $P > 0.05$). Coronary perfusion pressure in Group A increased already within the first 2 min of reperfusion (Figure 6), reflecting an increase in coronary resistance. In contrast, we saw an initial pressure decrease in both antithrombin-treated groups, corresponding to coronary dilatation. With ongoing reperfusion, coronary perfusion pressure rose significantly in all groups. However, the values for Groups B and D, i.e. those treated with antithrombin, did not exceed those of the time-matched controls without ischaemia at the end of the observation period (20 min). These time-control measurements without ischaemia also showed a slight increase in perfusion pressure, but with no difference between the control and antithrombin-treated

groups (min 20; Groups E and F: 79.0 ± 4.2 and 78.0 ± 2.7 cmH₂O, respectively).

Adding HES to the KHB perfusate reduced the post-ischaemic coronary pressure rise slightly in both non-treated and hearts pre-treated with antithrombin (Figure 6). However, this was only significant in the former (Group A vs. Group C). Thus, HES was unable to mimic the pressure lowering action of antithrombin.

3.4 Transudate formation

Transudate formation represents the direct measure of net fluid filtration in the intact coronary bed of the isolated heart and amounted to ~5% of coronary flow under basal conditions in all groups (A, 0.42 ± 0.07 ; B, 0.45 ± 0.08 ; C, 0.48 ± 0.06 ; D, 0.43 ± 0.09 ; E, 0.47 ± 0.5 ; and F, $0.45 \pm$

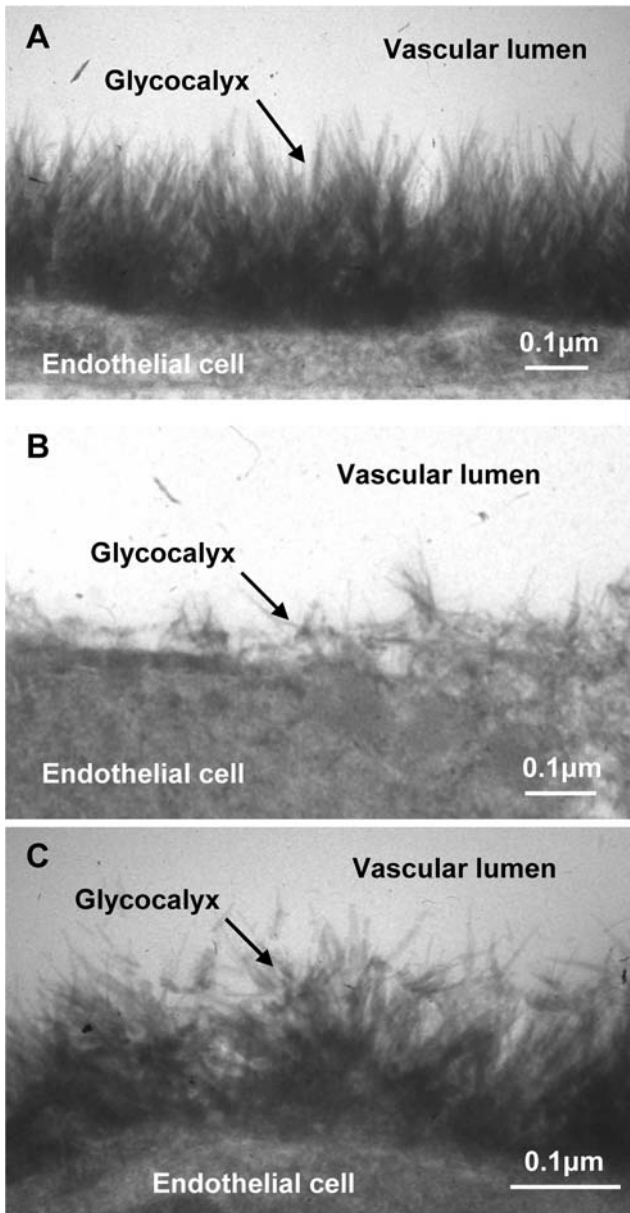


Figure 3 Electron microscopic views of the hearts stained to reveal the glycocalyx. (A) An intact glycocalyx after 20 min of perfusion (Group E) and (B) the nearly completely degraded endothelial glycocalyx after 20 min of warm ischaemia and consecutive reperfusion (Group A). (C) The glycocalyx after pre-treatment with antithrombin and 20 min of warm ischaemia and reperfusion (Group B). The endothelial glycocalyx is mostly intact.

0.06 mL/min/g; all comparisons $P > 0.05$). As transudate flow is pressure-dependent, it was normalized to the individually pertaining coronary perfusion pressure in all subsequent presentations.

In control Group A, transudate formation increased about three-fold within the first 2 min ($P < 0.01$) and then further (in total four-fold) during the course of reperfusion (Figure 7). Pre-treatment with antithrombin prevented the increase in coronary leak (Group B). Leak was also attenuated somewhat in the presence of colloid (HES Group C vs. Group A $P < 0.05$). Interestingly, the effects of antithrombin and colloid seemed to be additive, with Group D tending to have the lowest transudate value of all post-ischaemic hearts, with no difference to baseline values (Figure 7).

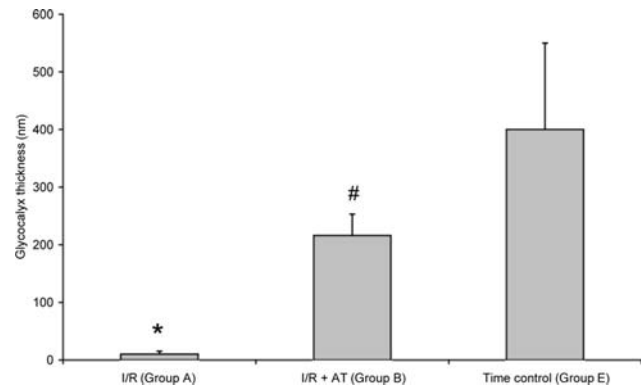


Figure 4 Thickness of the endothelial glycocalyx (nm, mean values \pm SD) measured at nine different sites in each of three hearts/group. *Group A vs. Groups B and E ($P < 0.01$). #Group B vs. Group E ($P < 0.05$). I/R, ischaemia/reperfusion; AT, antithrombin III.

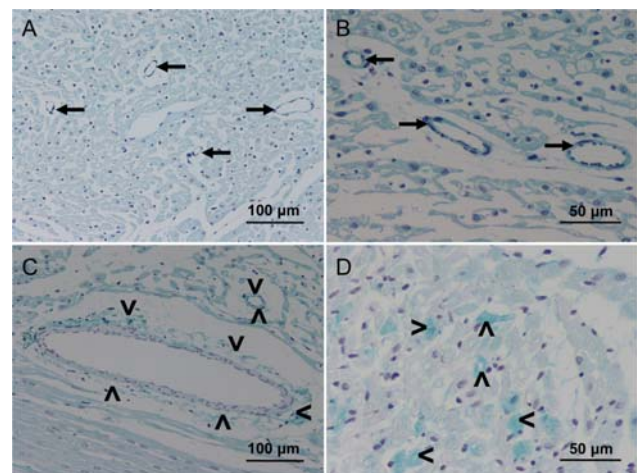


Figure 5 Immunohistological stainings revealing the distribution of antithrombin III after application to hearts with an intact (A and B) or a severely impaired (C and D) endothelial glycocalyx. In a healthy coronary vascular system, with an intact glycocalyx, infused antithrombin presents itself as a ring on the endothelial cells (arrow, Group F). This rather conspicuous zone indicates that antithrombin is located both on and within the glycocalyx. In vessels of hearts subjected to ischaemia/reperfusion, with a deteriorated glycocalyx, significant antithrombin staining is found in the interstitial tissue and not in the vessel when antithrombin is infused following the ischaemic damage (V symbol, no study group).

The final value of Group A was significantly higher than in all other groups ($P < 0.01$).

3.5 Extravasation of hydroxyethyl starch

Net colloid extravasation of HES was calculated as the product of transudate formation per gram heart weight and HES concentration in the transudate. Values are listed in Table 1. Hearts subjected to ischaemia without antithrombin (Group A) showed a large increase in HES filtration right from the beginning of reperfusion and filtration increasing further throughout the entire protocol ($P < 0.05$). Colloid net filtration in the antithrombin-treated Group B was significantly lower already at the onset of reperfusion ($P < 0.01$ vs. Group A at min 20; Table 1) and did not increase significantly over time.

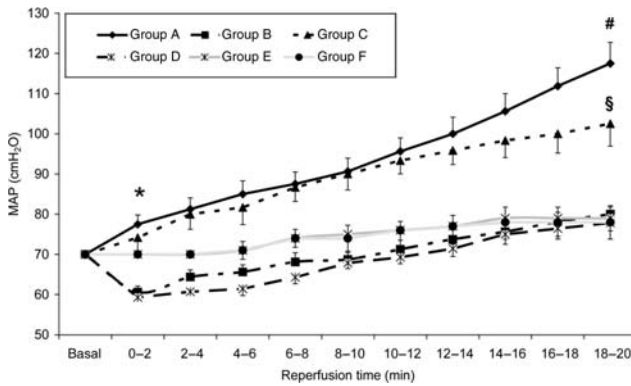


Figure 6 Mean arterial coronary perfusion pressure (MAP) in relation to time during reperfusion. *min 2: Group A vs. Groups B and D ($P < 0.05$); Group C vs. Groups B and D ($P < 0.05$). #min 20: Group A vs. Group C ($P < 0.05$) and Group A vs. Groups B, D, E, and F ($P < 0.01$). §min 20: Group C vs. Groups B, D, E, and F ($P < 0.05$).

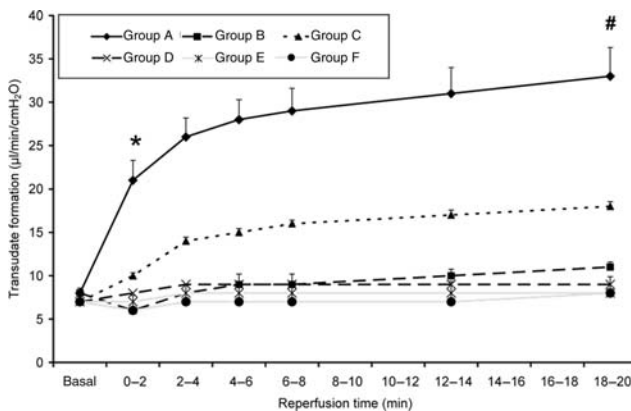


Figure 7 Transudate formation in relation to sampling period during reperfusion. Transudate flow was normalized to perfusion pressure. *min 2: Group A vs. Groups B-F ($P < 0.05$). #min 20: Group A vs. Group C ($P < 0.05$) and Group A vs. Groups B, D, E, and F ($P < 0.01$).

3.6 Measured tissue oedema

Mean wet-to-dry-weight ratios of isolated hearts were 7.1 ± 0.6 and 7.3 ± 0.8 measured after preparation and 45 min of perfusion with KHB with or without antithrombin treatment (time-control Groups E and F, respectively). The ratios were higher following I/R (Group A: 9.1 ± 0.3 and Group C: 8.6 ± 0.5 , $P < 0.05$ vs. Groups E and F), indicating a significant increase in tissue oedema formation. Application of antithrombin to the hearts before ischaemia produced significantly lower ratios (Group B: 7.9 ± 0.3 and Group D: 7.9 ± 0.5 , $P < 0.05$ vs. Group A), with no significant difference in oedema compared with time controls.

3.7 Rates of release of lactate, purines, and uric acid

Metabolic parameters of hearts subjected to I/R are listed in *Table 1*. Lactate release was identical in Groups A and B during equilibration. Release of lactate increased dramatically in both groups after the start of reperfusion ($P < 0.01$), and antithrombin pre-treatment having a slight beneficial effect ($P < 0.05$, Group B vs. Group A). The rate of lactate release decreased rapidly in both groups during reperfusion, returning to near basal values (*Table 1*).

Table 1 Comparison of metabolite release, permeability, and oedema

Parameter	Time (min)	Group A	Group B
Release of lactate ($\mu\text{mol}/\text{min}/\text{g}$)	Basal	1.65 ± 0.25	1.43 ± 0.31
	0'-5'	$11.50 \pm 0.84^*$	$7.92 \pm 1.09^*$
	5'-10'	2.56 ± 0.19	1.64 ± 0.22
	10'-15'	2.14 ± 0.17	1.42 ± 0.23
	15'-20'	1.95 ± 0.15	1.62 ± 0.25
Release of purines (nmol/min/g)	Basal	6.9 ± 0.9	9.9 ± 1.8
	0'-5'	$360.4 \pm 46.1^{*,\dagger}$	$217.6 \pm 34.9^*$
	5'-10'	$77.9 \pm 6.6^{*,\dagger}$	$51.0 \pm 12.4^*$
	10'-15'	$42.8 \pm 2.8^{*,\dagger}$	$20.3 \pm 4.7^*$
	15'-20'	$32.2 \pm 2.8^{*,\dagger}$	15.9 ± 4.4
Release of uric acid (nmol/min/g)	Basal	5.17 ± 0.80	4.39 ± 0.46
	0'-5'	$14.68 \pm 1.77^*$	$11.16 \pm 1.69^*$
	5'-10'	6.98 ± 0.56	7.02 ± 0.50
	10'-15'	6.44 ± 0.42	4.18 ± 0.56
	15'-20'	6.28 ± 0.40	3.94 ± 0.66
Extravasation of HES (mg/min/g)	Basal	n.d.	n.d.
	0'-5'	$8.44 \pm 1.42^\ddagger$	1.52 ± 0.36
	5'-10'	$11.76 \pm 2.31^\ddagger$	3.31 ± 0.56
	10'-15'	$14.98 \pm 2.70^\ddagger$	4.20 ± 0.51
	15'-20'	$18.47 \pm 1.40^\ddagger$	5.89 ± 0.69
Heart weight (wet-dry ratio)	End (20')	$9.12 \pm 0.25^\ddagger$	7.94 ± 0.27

Values are mean \pm SEM, $n = 8$ each. Group A, ischaemia without pre-treatment; Group B, ischaemia after pre-treatment with antithrombin III; basal, value before ischaemia under steady-state conditions; min 0-5, 5-10, 10-15 and 15-20, time after start of reperfusion; purines, sum of adenosine, inosine, hypoxanthine and xanthine; n.d., not detected.

* $P < 0.05$ vs. basal value.

$^\ddagger P < 0.05$, Group A vs. Group B.

Basal purine release was identical in Groups A and B. During the first minutes of reperfusion, purine release increased 20- to 60-fold ($P < 0.01$), but the rate in Group B was significantly lower than in Group A ($P < 0.05$) throughout the entire reperfusion (*Table 1*).

Uric acid was released in comparable concentrations in Groups A and B during equilibration. In the first minutes of reperfusion after ischaemia, we measured a considerable increase in uric acid release ($P < 0.05$), with rates comparable between both groups. After the peak, rates of release declined similarly to baseline values (*Table 1*). However, the ratios of the release rate of precursor purines to that of urate, a measure of oxidative stress, were significantly higher in Group A after ischaemia than in the antithrombin-treated hearts (Group B). After 20 min of reperfusion, the respective values were 5.1 and 4.0 ($P < 0.05$). High ratios result when urate is being consumed by oxidation.

4. Discussion

This study demonstrates that antithrombin has a stabilizing impact on the endothelial glycocalyx, preventing shedding following I/R. The preservation of this structure is associated with a maintained physiological endothelial

permeability barrier, lessened post-ischaemic rise in coronary resistance to flow, and mitigation of tissue oedema. It is also apparent that antithrombin penetrates into and binds to the coronary endothelial glycocalyx but does not seem to reach the interstitial space when applied to an intact glycocalyx (Figure 5). Thus, its protective action must be exerted directly at, or at least via, the endothelial glycocalyx.

The potential of antithrombin to protect the glycocalyx has already been shown by Chappell *et al.*¹³ following infusion of TNF- α in an isolated guinea pig heart model. Recent studies in humans have revealed that the degree of glycocalyx shedding depends on the extent of the ischaemic impact.¹⁸ Because this is also true for the intensity of septic shock,¹⁹ there obviously seem to be causal correlations between the severity of disease and glycocalyx integrity. In the isolated heart, 20 min of warm no-flow ischaemia have been shown to damage the vascular endothelium, degrading the entire glycocalyx.⁹ This results in an impressive increase in the coronary fluid leak, increased colloid extravasation, profound tissue oedema, and a significant rise in coronary perfusion pressure.⁹ These effects are also demonstrated in the present study. However, antithrombin pre-treatment produced significant improvements of these features, along with signs of lower metabolic and oxidative stress, suggesting secondary effects on the myocardial and endothelial cells beyond a protection of the glycocalyx. Endothelial cells and the glycocalyx together compose the vascular barrier function.¹² A strongly increased vascular leak can only occur if both structures are damaged. Therefore, one further effect of antithrombin might be an inhibition of endothelial gap opening.

Administration of antithrombin to pigs suffering from lipopolysaccharide-induced sepsis has been shown to significantly ameliorate vascular leakage.²⁰ Antithrombin inhibits numerous serine proteases such as tryptase, thrombin, or plasmin,²¹ and its application should abrogate proteolytic damage to cardiac tissue, including to the endothelial glycocalyx. Serine proteases participate in a wide range of functions in the body, such as blood clotting, immunity, and inflammation.²² The acute inflammatory response of I/R injury is associated with enhanced thrombin formation.^{23–25} Thrombin, in turn, activates platelets, endothelium, and leucocytes and forms fibrin from fibrinogen²⁶ and, thus, links the inflammatory and coagulation systems.²⁷ Escalation of thrombin formation may significantly contribute to the adverse functional consequences of ischaemia to myocardium,²⁸ whereas inhibition by antithrombin III should be protective.²⁹ Indeed, supplementation of antithrombin has alleviated I/R injury in the liver,³⁰ kidney,^{2,31} lung,³² and intestine.³³ However, this protection has been largely attributed to the anti-inflammatory effects of antithrombin, suggested to be based mainly on the release of prostacyclin mediated by antithrombin interaction with endothelial cell surface glycosaminoglycans.^{3,4} In our particular model, there is neither plasma nor platelets or inflammatory cells, so that antithrombin must be acting by some other mechanism here.

Resident mast cells are stores for numerous proteases (tryptase, chymase, mast-cell protease 5, heparanase activity, etc.). At this time, we do not exactly know which proteases are responsible for post-ischaemic shedding of the glycocalyx. Preliminary studies show increases in

tryptase and cathepsin B activity in the myocardium, the former being a typical mast-cell constituent. Previously, we have shown that hydrocortisone is able to preserve the glycocalyx, as it stabilizes mast cells, preventing their degranulation.⁹ Antithrombin, in contrast, remains within the vessel when the vascular barrier is intact without distributing into cardiac tissue (see immunohistology, Figure 5A and B). Accordingly, its mode of action must differ from that of hydrocortisone.

We hypothesize that stabilization of the glycocalyx may result from inhibition of enzymes at this location. Pertinently, in the presence of α -thrombin, a six-fold elevation of heparanase action has been reported.³⁴ Heparanase is an enzyme that cleaves heparan sulfate, thereby releasing growth factors that are implicated in tumour cell proliferation, metastasis, and angiogenesis.³⁴ Its bacterial analogue, heparinase, has recently been shown to selectively shed heparan sulfates from the endothelial glycocalyx.¹¹ Application of antithrombin III has resulted in complete inhibition of thrombin-mediated facilitation of heparanase action, despite basal activity of heparanase being unaffected.³⁴ Thus, a sufficient level of the serine protease inhibitor antithrombin within the glycocalyx may, by preventing the activation of thrombin or other serine proteases, prevent the consecutive shedding. Noteworthy in this context is that the blood concentration of antithrombin III falls by 30–40% in critically ill patients suffering from severe sepsis, the level correlating negatively with severity of disease.^{5,35} As we report here, when the glycocalyx is degraded, antithrombin is found predominantly in the interstitial tissue (Figure 5). This could have important consequences for the clinic, explaining why antithrombin levels are significantly reduced in patients with an impaired vascular barrier, e.g. suffering from ischaemia or sepsis.

Measurements of syndecan-1 and heparan sulfate, constituent parts of the glycocalyx, in the coronary effluent showed high concentrations following ischaemic insult (10- to 20-fold increases). Additionally, electron microscopy revealed a complete destruction of the glycocalyx as a component of damage resulting from I/R. Antithrombin applied before the ischaemic insult significantly decreased the shedding of the glycocalyx, as evidenced both by measurements of the components and by electron microscopy (Figures 2 and 3, respectively). Concerning function, action on the combined glycocalyx and endothelial cell barrier is better assessed in the presence of colloidal substances, which have been shown to be indispensable for the integrity of the glycocalyx.³⁶ The forces possibly affecting net fluid filtration in the present model are the intra- vs. extravascular pressures and the oncotic pressure.³⁶ With respect to the former, coronary vascular resistance increased significantly less in the hearts treated with antithrombin III, indicative of coronary dilatation and/or diminished obstruction. Thus, perfusion pressure may have extended further into the vascular bed, possibly elevating microvascular filtration. However, the converse effect prevailed in hearts treated with antithrombin, i.e. leak was reduced. Besides damage to the glycocalyx, some of the increased transudate flow seen after ischaemia may arise from gap formation between endothelial cells. Gap formation should increase hydraulic conductivity once the glycocalyx has been stripped away, even when the forces driving fluid extravasation are unchanged. Prolonged application of colloid may only

marginally affect fluid transport in such a situation, because oncotic equilibration occurs between the vascular and interstitial spaces.³⁶ Enhanced perfusate viscosity could explain the effect of HES on transudate flow (Figure 3C). The effect of HES on post-ischaemic coronary resistance (Figure 6) is more difficult to explain. The exact underlying mechanisms for increased coronary resistance during reperfusion are unclear, but possibilities are extramural compression, endothelial cell swelling, and a shedding-induced reduction of NO release which causes a relative vasoconstriction. Prolonged infusion of HES may alleviate such influences in the present model.

In the case of hearts pre-treated with antithrombin, the additional application of HES accentuated the protection further, as HES-treated hearts achieved the same low-leakage values during reperfusion as they did before ischaemia. It seems straightforward that the actions of a colloid on fluid filtration come to bear more strongly when the glycocalyx is intact.³⁶ This result thus further supports the conclusion that antithrombin preserves the glycocalyx.

Some aspects of the model used in this study need to be critically addressed. Constant flow instead of constant pressure perfusion was chosen for the present experiments in order to guarantee constant levels of infused substances, e.g. antithrombin and HES. A methodological peculiarity of the isolated heart model is the fact that perfusion with artificial media for >30 min causes tissue oedema even in undamaged hearts. The addition of colloidal substances such as HES helps to alleviate this if the endothelial glycocalyx is intact.^{9,12,14} Preventing oedema formation should forestall elevation of extramural pressure, i.e. elevation of coronary resistance is largely insensitive to vasodilators. Furthermore, since the isolated hearts are perfused with a blood-free KHB solution, it is impossible to assess aspects of I/R injuries which may be initiated and/or influenced by plasma-borne factors and formed constituents of blood. On the other hand, physiological antioxidants of plasma are absent, perhaps accentuating damage by reactive oxygen species. Furthermore, the Langendorff-heart preparation per definition excludes performance of pressure-volume work. Thus, the recovery of myocardial pump performance was not directly assessable in the present model.

A previous study perfusing isolated rat hearts for 30 min after global ischaemia (20 min, 20°C) showed only minor impacts of antithrombin on I/R injury.³⁷ However, hypothermia of 20°C is cardioprotective in itself. The measured coronary flow was significantly reduced after I/R, an effect partly inhibited by antithrombin. This is in accordance to our study, where coronary perfusion pressure increased significantly more in the control group during flow constant perfusion. Interestingly, Margreiter *et al.*³⁷ revealed that high doses of antithrombin (8 vs. 1 U/mL) might worsen tissue injury. Therefore, we chose to apply 1 U/mL, which corresponds to physiological levels in human blood.

Known cardioprotective measures such as hypothermia and staged reperfusion minimize oxidative stress and should also protect the glycocalyx. Lowering temperature slows down proteolytic enzymes. Managing reperfusion has the potential to attenuate oxidation-mediated activation of matrix metalloproteases. It lessens activation of myocardial mast cells, as do membrane-stabilizing agents. Thus, all of these interventions should result in less shedding of the endothelial glycocalyx.

We conclude that antithrombin has a multifactorial impact on the post-ischaemic, reperfused heart. Penetration and preservation of the endothelial glycocalyx, and the consequential maintenance of the vascular barrier with prevention of interstitial oedema, imply a great potential in the therapy of I/R injury. Although several studies indeed have shown antithrombin III to reduce I/R injuries, studies on patients undergoing major operations associated with tissue ischaemia and reperfusion are necessary to substantiate our findings. Antithrombin application has previously reduced mortality of patients suffering from severe sepsis. In this context, preservation of the glycocalyx by antithrombin seems a likely aspect of action.

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